Cotinine formation by cDNA-expressed human cytochromes P450

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Introduction: Nicotine, the major alkaloid in tobacco, is extensively metabolised in the liver and to a smaller extent in the lung. Nicotine is primarily converted to cotinine and nicotine-1'-*N*-oxide by *C*- and *N*-oxidation respectively. The metabolic transformation of nicotine to cotinine in mammals is a two-step reaction. The first step is the catalysis of nicotine to nicotine-$\Delta^{1'(5')}$-iminium ion by the microsomal cytochrome P450 system [1]. The conversion of the iminium ion to cotinine is mediated by cytosolic aldehyde oxidases [2].

Since nicotine has been implicated in maintaining the use of tobacco products, an understanding of the role of the specific enzyme systems involved in the metabolism of nicotine in humans is required. Although it is unclear which human P450 isozymes are responsible for the metabolism of nicotine to cotinine, phenobarbitone pre-treatment is associated with increased cotinine formation [3]. Recent work using a HepG2 cell lysate cDNA expression system has demonstrated that cells which express CYP2B6 exhibit the highest rate of C-oxidation of nicotine [4].

We have studied a cDNA-directed expression system, in which human B-lymphoblastoid cell lines, engineered to express stably individual human cytochromes P450 cDNA, was used to determine which P450 isozymes are responsible for the metabolism of nicotine to cotinine. In order to study the metabolism of nicotine *in vitro* we have used either an NADPH-generating system or cumene hydroperoxide. At low concentrations, cumene hydroperoxide exploits the ability of CYP2D6 to function as a peroxygenase and has been described as being isozyme-specific for CYP2D6 [5].

Materials and methods: Incubations for the determination of nicotine C-oxidation activity were in a final volume of 500 µL of 0.1 M phosphate buffer pH 7.4 at 37°C. Microsomal protein (10–100 µg) was incubated with nicotine (1 mM). NADPH (100 µM) and $MgCl_2$ (10 mM). Reactions were started by the addition of 1 mM nicotine (50 µL of 10 mM stock) and stopped after 20 min by the addition of 5 M NaOH (50 µL).

When the NADPH system was replaced by cumene hydroperoxide, incubations were carried out in a final volume of 100 µL (0.1 M phosphate buffer pH 7.4) at 25°C. CuOOH (80% in cumene) was diluted first to 40 mM in 50% methanol in H_2O (v/v) and then to 1.25 mM in 0.1 M phosphate buffer pH 7.4. An aliquot (10 µL) of this solution was added to the microsomal suspension before the addition of nicotine (1 mM), and the reaction was stopped by the addition of 5 m NaOH (50 µL). As a consequence of spontaneous hydrolysis, incubations containing no protein were carried out in parallel.

For the inhibition studies incubations were conducted in the presence of quinidine (10 µM), a specific inhibitor of CYP2D6 [6]. Microsomal protein (50 µg) was incubated with nicotine (1 mM). Control incubations without inhibitor were

Table 1: Cotinine formation by cDNA-expressed human cytochrome B-lymphoblastoid cell lines in the presence of NADPH (means ± SD; n = 5).

P450 isozyme	Cotinine formation (pmol min^{-1} mg^{-1})	+ Quinidine	% Inhibition
Control	0	–	–
2A6	1.47 ± 1.06	–	–
2B6	5.52 ± 3.2	5.62 ± 1.7	0
2C8	0	–	–
2C9	0	–	–
2D6	2.26 ± 4.81	0	100
2E1	0	–	–
3A4	0	–	–

Table 2: Cotinine formation by cDNA-expressed human cytochrome B-lymphoblastoid cell lines in the presence of cumene hydroperoxide (means ± SD; n = 8).

P450 isozyme	Cotinine formation (pmol min^{-1} mg^{-1})	+ Quinidine	% Inhibition
Control	5	–	–
2A6	15.5 ± 27	–	–
2B6	273 ± 112	275 ± 49	0
2C8	0	–	–
2C9	0	–	–
2D6	78.2 ± 38	0	100
2E1	0	–	–
3A4	0	–	–

Dichloromethane (1 mL), containing the internal standard, *N*-ethyl norcotinine (10 µL; 5 mg L^{-1}), was added and the samples were mechanically shaken (5 min). After centrifugation (3,000 rpm: 5 min) the aqueous layer was removed and discarded and the organic layer placed in a tapered glass vial (0.8 mL). The sample was evaporated to dryness, the residue redissolved in methanol (10 µL) and an aliquot (1 µL) injected onto the gas chromatograph.

The gas chromatograph was a Hewlett-Packard Model 5890II instrument equipped with a nitrogen-phosphorous detector. Analyses were performed on a fused silica capillary column (10 m × 0.32 mm ID). The injection port was maintained at 250°C and the detector at 280°C. The column temperature was increased from 100–220°C at 30°C min^{-1}, maintained at 220°C for 5 min, increased from 220–270°C at 10°C min^{-1} and then maintained at 270°C for 5 min. The carrier gas (helium), hydrogen, air and make-up gas (nitrogen) were used at flow rates of 2.5, 10, 120 and 10 mL min^{-1}, respectively. Analyses were performed using the splitless injection technique and under these conditions nicotine, cotinine and *N*-ethyl norcotinine had retention times of 3.9, 7.1 and 7.3 min respectively.

Results: Tables 1 and 2 show that those cells which expressed CYP2B6 had the highest nicotine *C*-oxidation activity in the presence of both NADPH and cumene hydroperoxide.

CYP2A6 and CYP2D6 also appeared to be capable of mediating the conversion of nicotine to cotinine in the presence of either oxidant, although the activity seen was considerably less than that with CYP2B6. Cells expressing CYP2C8, 2C9, 2E1 or 3A4 showed a lack of nicotine *C*-oxidation activity in the presence of either NADPH or cumene hydroperoxide.

Quinidine has the ability to inhibit the CYP2D6-mediated conversion of nicotine to cotinine completely. However, it has no inhibitory effect on the CYP2B6-mediated *C*-oxidation of nicotine (Tables 1 and 2).

Discussion: It is now well established that inter-individual variability in the expression of cytochromes P450 has considerable pharmacological and toxicological consequences. In this study three P450 isozymes, each with known variability in expression in human liver samples [7], have been shown to mediate the conversion of nicotine to cotinine. With respect to CYP2B6, the results obtained are in agreement with those of Flammang *et al.* [4] and indicate that this isozyme is highly active in the conversion of nicotine to cotinine. However, it is important to note that CYP2B6 is not constitutively expressed in human liver and as such will be relatively unimportant in the metabolism of nicotine in most individuals.

The ability of quinidine (a specific inhibitor of CYP2D6) to inhibit the conversion of nicotine to cotinine mediated by CYP2D6, and not CYP2B6, confirms the role of this isozyme in the *C*-oxidation of nicotine. However, cumene hydroperoxide was not specific for the CYP2D6-mediated conversion of nicotine to cotinine in these studies.

As a consequence of its polymorphic expression in humans, CYP2D6 has been extensively studied. 5–10% of a Caucasian population exhibit a defect in the gene which codes for this enzyme, which renders them poor metabolisers of many widely used xenobiotics. Although less is known of the mechanisms that contribute to variability in the expression of CYP2A6 in human liver specimens, an inactive variant of this enzyme has been identified.

Unlike CYP2B6, both CYP2A6 and CYP2D6 are constitutive enzymes of human liver and as such are more likely to contribute to the C-oxidation of nicotine in humans. Inter-individual variability in the expression of these enzymes may have important implications for an individual's ability to metabolise nicotine to cotinine.

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